Mobility of Fluorescent Derivatives of Cytochrome c in Mitochondria

JANE M. VANDERKOOI, GRZEGORZ MANIARA, and MARIA ERECINSKA
Department of Biochemistry and Biophysics and Department of Pharmacology, School of Medicine, University of Pennsylvania, Philadelphia 19104

ABSTRACT Motion of cytochrome c bound to giant (2–10-μm diam) mitochondria isolated from the waterbug *Lethocerus indicus* was examined using the technique of fluorescence recovery after photobleaching. Fluorescent cytochrome c was exchanged for native cytochrome c through partly damaged outer membrane. Recovery profiles were not statistically different when the fluorescence from iron-free cytochrome c or fluorescein-labeled cytochrome c was used and were essentially the same in the presence or absence of an uncoupler. In the presence of excess porphyrin cytochrome c, the apparent diffusion coefficient was 6×10^{-11} cm^2/s in 0.3 M sucrose-mannitol-EDTA and 3×10^{-10} cm^2/s in 0.10 M KCl/0.10 M sucrose. At concentrations of porphyrin cytochrome c that are stoichiometric with cytochrome c oxidase and for mitochondria in which excess cytochrome c was washed away, two components were observed in the recovery profile. The diffusion coefficient of the fast component was 1×10^{-10} cm^2/s. The second component showed no recovery during the time scale of measurement ($D < 10^{-12}$ cm^2/s). We speculate on the origin of the immobile fraction.

Cytochrome *c* is a small extrinsic membrane protein that transfers electrons between two large intrinsic complexes, cytochrome *c* oxidase and cytochrome bc_1 (for review, see Dixit and Vanderkooi [5]). Free diffusion of cytochrome *c*, either laterally on the membrane or in the intermembrane space, could accomplish this shuttle. Measurement of the lateral diffusion of cytochrome *c* by fluorescence recovery after photobleaching (FRAP)[1] in two laboratories has suggested that cytochrome *c* is freely diffusible (11, 14), but the interpretation of the data is a matter of controversy. The first group suggested that the rates of cytochrome *c* diffusion are compatible with its role as a mobile electron carrier (11) whereas the second group interpreted their results in terms of a dynamic aggregate between cytochrome *c* and its redox partners (14). However, cytochrome *c* that is covalently attached to the membrane can transfer electrons (7), and cross-linked cytochrome *c* in glutaraldehyde-treated mitochondria is also active (27). In addition, the existence of high-affinity binding sites ($K \leq 10^{-7}$) on both the oxidase (8) and reductase (3) argues against the diffusion of free cytochrome *c* as a necessary prerequisite for electron transfer.

In this paper we reinvestigate the question of the mobility of cytochrome *c* bound to mitochondria using the FRAP technique. Our experiments differ from other work in the following aspects: (*a*) Giant mitochondria, isolated from the waterbug, *Lethocerus indicus*, were used; these mitochondria are naturally large without preliminary manipulations of animals that involve toxic treatment with drugs. (*b*) The preparation contained a population that were somewhat swollen and had the outer membrane only partially attached. (*c*) The iron-free derivative ("porphyrin" cytochrome *c*) was used; the intrinsic porphyrin of this derivative has high fluorescence quantum yield which permits examination of low concentrations. It has previously been shown that porphyrin cytochrome *c* is a competitive inhibitor of the native protein (28) with an inhibitory constant about the same as the binding constant for native cytochrome (30, 31); since the inhibition is competitive, it can be assumed that the inactive derivative binds at the same site as the native cytochrome *c*. (*d*) Concentration of added porphyrin cytochrome *c* was varied and "washed" mitochondria with tightly bound cytochrome *c* were examined.

MATERIALS AND METHODS

Type III and type VI horse heart cytochrome *c*, fluorescein isothiocyanate, and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). Iron-free

[1] *Abbreviations used in this paper:* DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; FRAP, fluorescence recovery after photobleaching; MSE, 0.225 M mannitol, 0.075 M sucrose, and 0.002 M EDTA.

THE JOURNAL OF CELL BIOLOGY · VOLUME 100 FEBRUARY 1985 435-441
© The Rockefeller University Press · 0021-9525/85/02/0435/07 $1.00

porphyrin cytochrome *c* was prepared and purified on Sephadex and CM-cellulose as previously described (28). Further purification was accomplished by chromatography on Sephadex G-50-120 column (1 × 20 cm). The preparation contained no dimerized form of protein. DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate) was obtained from Molecular Probes, Inc. (Junction City, OR). Fluorescein-labeled cytochrome *c* was prepared according to Hochman et al. (14). Other chemicals were the highest purity commercially available. Water was deionized and distilled.

Oxygen uptake was measured with a Clark type oxygen electrode at 25°C. Protein was measured using the biuret reaction with BSA as the standard (10). The concentration of porphyrin cytochrome *c* was estimated by using 1.5 as millimolar extinction coefficient at 620 nm (28).

Preparation of Mitochondria: Giant mitochondria were isolated from flight muscle of the waterbug, *Lethocerus indicus*. The insects were obtained from Thailand. Flight muscle tissue (0.15–0.25 g) was gently homogenized in 15 ml of isolation medium containing 0.15 M KCl, 0.0015 M EGTA, and 1% BSA (pH 7.1). The homogenate was filtered through four layers of cheesecloth which was wet with isolation medium. The filtrate was centrifuged at 80 *g* for 5 min. The pellet was discarded and the supernatant was centrifuged at 3,600 *g* for 5 min. The pellet was resuspended and then recentrifuged twice in the isolation medium and finally resuspended in 2 ml of buffer containing 0.225 M mannitol, 0.075 M sucrose, and 0.002 M EDTA (MSE), pH 7.1. In Fig. 3 the resuspension medium included KCl as indicated in the legends. All procedures were carried out in a water-ice bath.

Spectral Studies: Mitochondria (~2 mg protein/ml) were suspended in 50 mM phosphate buffer, pH 7.4, containing 1% sodium deoxycholate, and their spectral properties were measured in an Aminco DW2A double beam/split beam spectrophotometer (American Instrument Co., Inc., Silver Springs, MD). The oxidized base-line was obtained after addition of 1 mM K-ferricyanide to both the sample and the reference cuvettes whereas the reduced spectrum was recorded after addition of sodium dithionite to the sample cuvette. The difference between the reduced (experimental) and oxidized (reference) samples was recorded.

Labeling Mitochondria with Fluorescent Derivatives of Cytochrome c: Mitochondria (1.5–2 mg protein/ml) were incubated with the cytochrome *c* derivatives for 15 min in 0.3 osmol solutions at room temperature. In cases where KCl was increased, we maintained the osmolarity by decreasing the amounts of MSE. These suspensions were used for the "unwashed" samples in the FRAP measurements. In the "washed" samples excess of the derivatives of cytochrome *c* was removed by centrifugation for 5 min at 3,600 *g*. The pellet was resuspended in the same medium that was used for incubation.

Fluorescence Photobleaching: The fluorescence photobleaching apparatus used was designed along conventional lines (15). A 2-W Argon-ion laser from Coherent Inc. (Palo Alto, CA) serves as the light source. The line at 514.5 nm was used. The microscope is a Leitz Ortholux II equipped for ploem illumination. Separation of the illuminating beam from the bleaching beam is accomplished by the beam-splitting approach between glass flats (19). A fast-acting shutter (Uniblitz, Vincent Associates, Rochester, NY) is positioned between the two optical flats. A small hole drilled in the shutter mounting passes the weak beam while the closed shutter blocks the main beam. The shutter is actuated by an Apple II microcomputer which also drives a second Uniblitz shutter placed in front of the detector. The two shutters are driven asynchronously so that the photomultiplier is not exposed to saturating light levels. After the beam splitter, the light is raised to the level of the microscope's ploem entrance and a biconvex lens ($f = 5$ cm) focuses the beam to a waist on the secondary image plane of the microscope which is conjugate to the in-focus specimen plane. An EMI 9865 B (EMI-Gencom, Inc., Plainview, NY) photomultiplier is mounted above the vertical output of the trinocular head and a Schott 590 OG filter (cutoff below 590 nm; Schott Optical Glass Inc., Dwryea, PA) is interposed to block the reflected incident radiation and pass the fluorescence. The photomultiplier is mounted in a cooled housing (FACT 50 MK III Cooler [EMI-Gencom Inc.]) which allows the detector to be cooled to −30°C. The high voltage supply (3,000 V), amplifier/discriminator (AD 100), and counter/accumulator (C-10) were all purchased from EMI Gencom, Inc. The system was used in photon counting mode. The BCD output from the C-10 counter is interfaced to the Apple II computer. A precision clock card (Mountain Hardware, Scotts Valley, CA) provides the Apple II with the high-precision time base.

We determined the spot size by using the convolution scan method on a film of DiI in collodion as described by Schneider and Webb (23). The $1/e^2$ intensity radius was 1 μm. The power of the unattenuated beam at 514.5 nm was 40–60 mW.

A recovery of fluorescence could be due to chemical recovery rather than diffusion. We thus conducted two experiments to rule this out for porphyrins. In one, protoporphyrin IX was immobilized in collodion as described above for DiI. No recovery was observed after bleaching. In the second, porphyrin cytochrome *c* solution was dripped onto a coverslip. The solution was dried in a desiccator. No recovery was observed after bleaching the dried porphyrin cytochrome *c*.

Analysis of Data: Under conditions of low values of bleach, a FRAP recovery curve is described by

$$F(t) = \frac{F(0) + F(\infty)(t/t_{1/2})}{1 + (t/t_{1/2})}, \quad (1)$$

where $F(0)$ is the fluorescence intensity at initial time of bleach and $F(\infty)$ is the intensity at time sufficient for total recovery (34).

Barisas and Leuther (2) and Yguerabide et al. (34) have pointed out that when the function

$$R(t) = F(\infty)/[F(\infty) - F(t)] \quad (2)$$

is plotted as a function of time, a straight line is obtained. From the intercept and slope the half-time, $t_{1/2}$, of recovery can be obtained:

$$\text{intercept/slope} = t_{1/2}. \quad (3)$$

This is related to the diffusion coefficient according to

$$t_{1/2} = \beta r^2/4D, \quad (4)$$

where r is the radius of the spot size, D is the diffusion coefficient, and β is a constant that depends upon the percent of bleach (1, 2, 34).

Our procedure to analyze the data was as follows. The data curves were linearized by plotting the $R(t)$ as a function of t. Usually about 30 data points were taken. The computer calculated a least squares fit to the data and values for $t_{1/2}$ and $F(0)$ were obtained. The data were replotted according to Eq. 1 and the calculated curves using the parameters derived from the linearization were superimposed. In some cases it was apparent that the choice of $F(\infty)$ was incorrect. In an iterative procedure, $F(\infty)$ was varied, the values for $t_{1/2}$ and $F(0)$ were recalculated, and simulated curves were reobtained. When satisfactory fit was obtained Eq. 4 was used to obtain the diffusion coefficient, D, using β values given by Yguerabide et al. (34). Data from successive bleaches were analyzed for analysis of variance (ANOV, F) by standard methods (35).

RESULTS

Size and Oxidative and Spectral Properties of Flight Muscle Mitochondria

Table I shows the rates of oxygen uptake of flight muscle mitochondria oxidizing three different substrates with (state 3) and without (state 4) ADP. It can be seen that the highest respiratory activity was attained with succinate whereas malate + glutamate was oxidized at a rate four- to sixfold slower. α-Glycerophosphate was a poorer substrate than succinate but better than malate (+ glutamate). These substrate specificities are characteristic of muscle mitochondria. The respiratory control ratios for the three substrates were 1.7, 1.8, and 2.7, respectively. The ADP/O ratios were 1.6 for succinate, 1.7 for α-glycerophosphate, and 2.8 for malate (+ glutamate). In all the experiments described above, the assay medium contained

TABLE I

Respiratory Activity of Mitochondria

Substrate	Oxygen consumption	
	−ADP	+ADP
	nmol/min per mg protein	
Succinate	75 ± 5	125 ± 7
α-Glycerophosphate	24 ± 3	43 ± 5
Malate (+) glutamate	12 ± 2	32 ± 3

The incubation medium contained: 0.225 M mannitol, 0.075 M sucrose, 0.002 M EDTA, 0.005 M KCl, 0.005 M NaP_i, 0.2% BSA, 0.01 M substrate, and 0.5–1 mg of mitochondrial protein (pH = 7.4). ADP, where indicated, was added at 0.3–0.6 μM. Values are means ± SD for three experiments.

0.2% BSA. In the absence of albumin, respiration in state 4 was increased, with no change in state 3, which resulted in smaller respiratory control ratios.

The cytochrome content of the mitochondria was estimated by absorption spectroscopy. The overall properties of the spectra were similar to those of other mitochondrial preparations: there were prominent peaks at 551, 562, and 605 nm characteristic of cytochrome *c*, *b*, and *a*, respectively. The concentration of cytochrome aa_3 was 0.85 nmol/mg of protein and the relative ratios of the cytochromes were within the range of values measured for mitochondria from other sources.

The flight muscle mitochondria suspended in MSE buffer showed oxygen uptake with 10 mM ascorbate that was greatly enhanced by the addition of 1 μM cytochrome *c*. Since the outer mitochondrial membrane of intact organelles is essentially impermeable to exogenous cytochrome *c* (21, 32), this observation means that the outer membrane of the giant mitochondria was partially damaged and permeable to added cytochrome *c*.

To further characterize the preparation, we examined the mitochondria under a light microscope (Fig. 1). In isotonic MSE medium the size of the diameter varied from 2 to 10 μm and occasionally was up to 20 μm (Fig. 2*A*). When the medium was diluted by one third with distilled water we observed that the small (i.e., 2 μm) mitochrondria expanded suddenly to ~5–20 μm and then did not change in size. By using dark-field optics, we saw that the outer membrane was disrupted but still attached to the inner membrane (Fig. 1*B*). The mitochondria suspended in the diluted MSE fragmented within 30–90 min and thus proved unsatisfactory for routine FRAP experiments. Therefore, in all our measurements, we used only those organelles that were at least 5 μm-diam and usually $\geq$10 μm. It was assumed, in agreement with the accessibility of mitochondria to exogenous cytochrome *c* described above, that the population of largest mitochondria which was isolated and maintained in the MSE buffer represented that of swollen organelles.

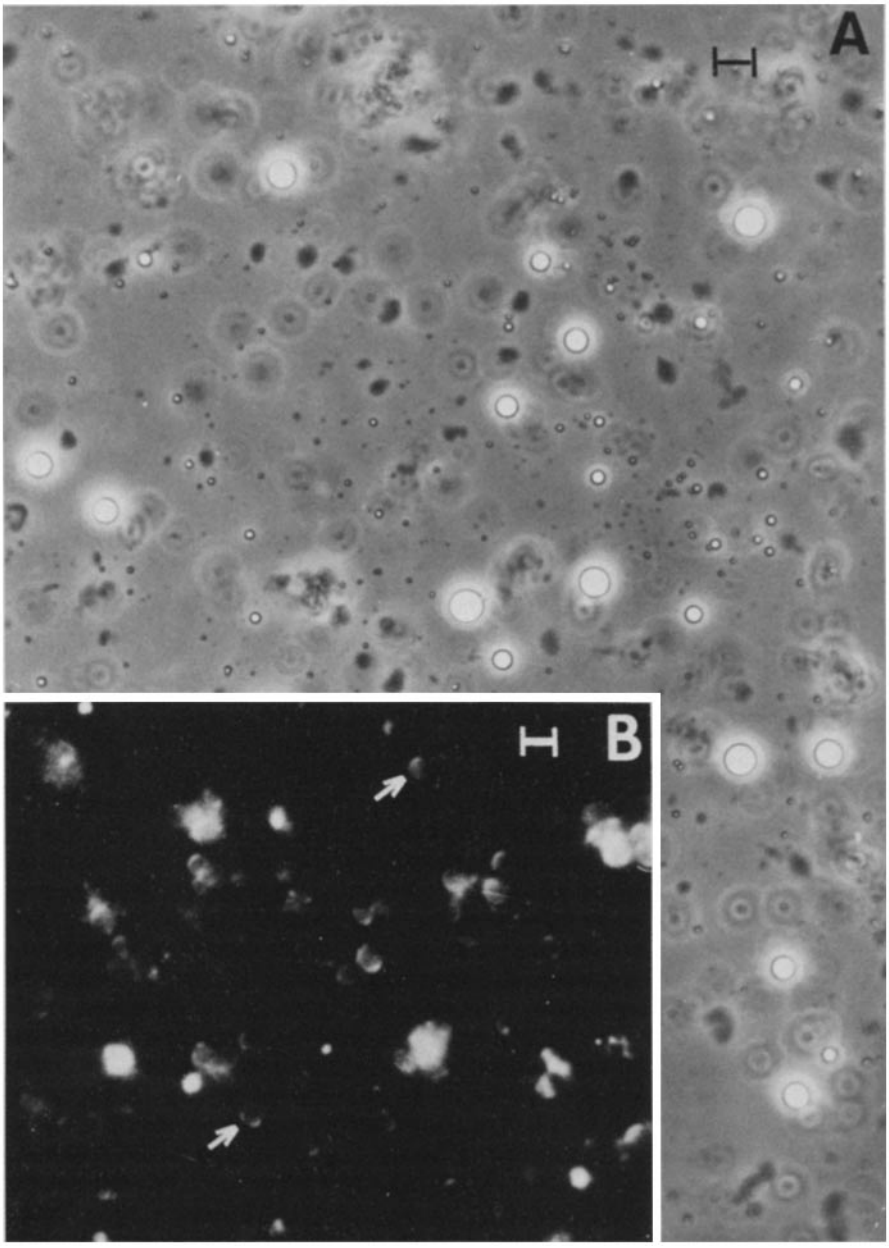

FIGURE 1 Micrograph of mitochondria. (*A*) The mitochondria were observed using a Zeiss standard microscope equipped with phase-contrast optics. The objectives were oil immersion plane achromate 100:1.25. (Under these optical conditions, the objects that are in focus have a halo around them.) Photographs were taken with a 35-mm camera. The medium was isotonic MSE. (*B*) Dark-field illumination was used. The MSE was diluted by one third with water. The arrows indicate two mitochondria in which the double membrane can be observed. Bars, 10 μm.

FRAP Profiles for Porphyrin Cytochrome c

The FRAP profiles for porphyrin cytochrome *c* in the presence of the giant mitochondria were examined as a function of porphyrin cytochrome *c* concentration. At high concentrations (20–80 μM) in which the stoichiometry of added porphyrin cytochrome *c* to cytochrome *c* oxidase was greater than 5:1, the recovery was 100% complete and the mean diffusion coefficient was 6.2×10^{-11} cm²/s in MSE, independent of porphyrin cytochrome *c* concentration. At low concentrations of porphyrin cytochrome *c*, the apparent diffusion coefficient appeared to be somewhat higher than for high concentrations and recovery was only ~50% (Fig. 2). The diffusion coefficients and recovery percentages are summarized in Table II.

The recovery profiles for fluorescein-labeled cytochrome *c* were obtained in several experiments (data not shown). We found no difference between fluorescein-cytochrome *c* and porphyrin cytochrome *c* in the recovery at concentrations that were in excess of the oxidase (>5:1 cytochrome *c* to oxidase). The detection of fluorescence by our instrument was not sensitive enough to measure lower concentrations of the fluorescein derivative. In some experiments we added the uncoupler, 1799 (2,6-dihydroxy 1,1,1,7,7,7, hexafluoro-2,6-*bis*[trifluoromethyl]-heptanone-4 *bis*[hexafluoroacetonyl] acetone; 10 μM). We could see no difference in the recovery curves in the presence and absence of uncoupler.

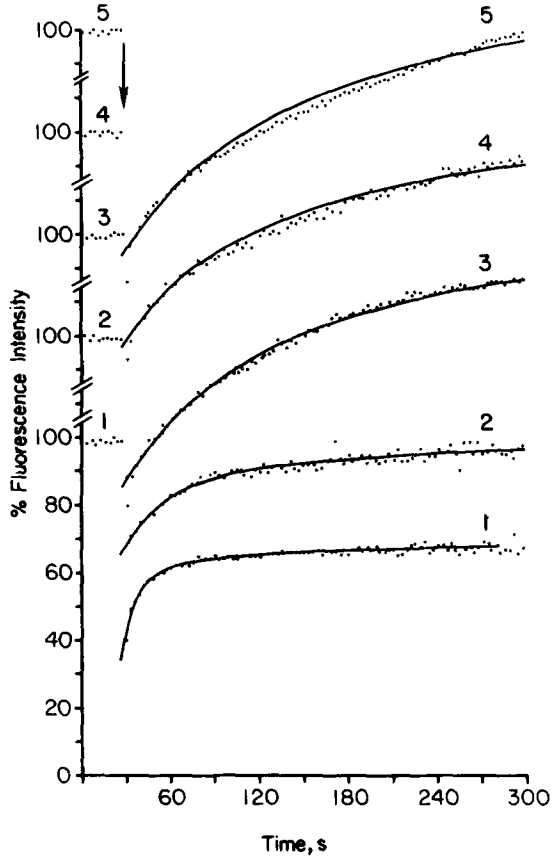

FIGURE 2 FRAP profiles as a function of added porphyrin cytochrome c. Mitochondria (2 mg protein/ml; ~2 μM in cytochrome a) in MSE were incubated for 15 min with the following concentrations of porphyrin cytochrome c: (*1*) 5 μM; (*2*) 10 μM; (3) 20 μM; (*4*) 50 μM; or (5) 80 μM. Arrow indicates the time when the bleaching beam was applied for 0.5 s. The points represent the experimental values and the solid lines are the computer best fit which gave these values for *D*: 2.2×10^{-10}, 1.1×10^{-10}, 3.4×10^{-11}, 2.2×10^{-11}, and 5.8×10^{-11} cm²/s for *1–5*, respectively, and for apparent recovery: 48, 51, 97, 94, and 99% for *1–5*, respectively.

TABLE II

Effect of Porphyrin Cytochrome c Concentration on FRAP Parameters

Concentration*	N‡	Recovery§	D‖
μM		%	cm²/s
80	5	97 ± 3	$6.4 \pm 1.5 \times 10^{-11}$
50	2	93 ± 3	$7.1 \pm 0.3 \times 10^{-11}$
40	2	99 ± 1	$6.5 \pm 1.6 \times 10^{-11}$
20	23	96 ± 2	$6.2 \pm 0.2 \times 10^{-11}$
10	5	57 ± 6	$1.5 \pm 1.3 \times 10^{-10}$
5	2	55 ± 10	$2.4 \pm 1.6 \times 10^{-10}$

* Mitochondria were prepared as described in Materials and Methods and then labeled with porphyrin cytochrome c by incubation in the concentrations indicated in the table for 15 min at room temperature. FRAP measurements were done as described in the legend of Fig. 2.
‡ Number of FRAP measurements taken.
§ Mean percent recovery ± SD.
‖ Diffusion coefficient ± SD.

Despite the problems of stability of the mitochondria in hypotonic medium, we also attempted to examine the FRAP profiles of porphyrin cytochrome *c* in mitochondria in MSE that was diluted by one third with water. We saw no difference in the recovery profiles for either low (5 μM) or high concentrations (50 μM) per 2 mg of mitochondrial protein between these swollen mitochondria and those examined above (Fig. 2 and Table II).

Effect of KCl on Binding and FRAP Profiles of Porphyrin Cytochrome c to Mitochondria

KCl dislodges bound porphyrin cytochrome *c* from the membrane (Fig. 3). This is well known and is indicative of the ionic binding of cytochrome *c* with the membrane (5).

Recovery of fluorescence after bleaching was faster after addition of KCl in the presence of excess porphyrin cytochrome *c* (Table III). The diffusion coefficient in the absence of KCl was 6.2×10^{-11} cm²/s and 33×10^{-11} cm²/s with 50 mM KCl (Table III). The effect of KCl is consistent with the off-rate that determines the apparent diffusion coefficients. This is discussed later.

In washed mitochondria the apparent diffusion coefficient was somewhat higher than in unwashed mitochondria at the same ionic strength (Table III). A higher diffusion coefficient was also observed at low porphyrin cytochrome *c* concentrations (Table II) in agreement with the results obtained on washed mitochondria. Analysis of variance revealed that there is no significant difference between different preparations of mitochondia.

FRAP Profiles for Consecutive Bleaches

In the experiments taken at low porphyrin cytochrome *c* concentrations, 100% recovery may not have been achieved because the sample was too extensively bleached. However, the observation that the percent recovery was nearly independent of the bleaching time and extent of the total bleach provides evidence that this is not a case. Further indication that the sample was not over-bleached was obtained from consecutive bleaches of the same spot. FRAP profiles for consecutive bleaches of washed mitochondria are shown in

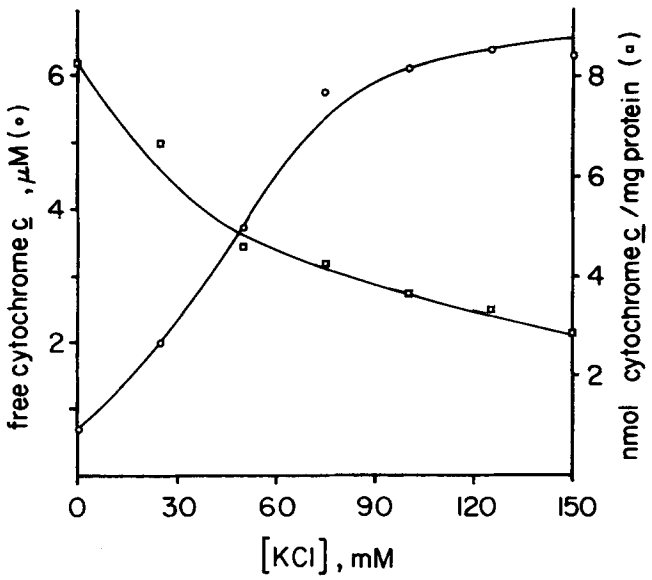

FIGURE 3 Binding as a function of KCl. Mitochondria (1.8 mg/ml) were incubated with 20 μM porphyrin cytochrome c in MSE for 15 min, followed by centrifugation at 3,600 g for 5 min. The pellet was resuspended in KCl concentrations indicated in the figure and MSE to give 0.3 osmol. After a 15-min incubation, the sample was centrifuged for 5 min at 3,600 g. The pellet was resuspended in the same medium, incubated for 15 min, and recentrifuged. The amounts of porphyrin cytochrome c in the pellet and the two supernatants were determined by the fluorescence intensity at 620 nm. The values for "free" in the figure indicate the sum of the two supernatants.

Fig. 4. We can see that on the first bleach the recovery was 50% but by the third bleach the recovery was 80% of the intensity obtained after recovery from the second bleach. This would indicate that the immobile phase did not recover on this time scale and the contribution of the "mobile" molecules of cytochrome *c* was relatively larger after the second and further bleaches. It also suggests that the "unrecovered" fluorescence cannot be accounted for by over-bleaching and that immobilization was not due to a laser effect which cross-linked a mobile fraction, since in these cases the percent recovery would be expected to be the same each time (16).

Lipid Mobility in Giant Mitochondria

The lipid probe DiI was also examined for lateral diffusion. We found that the recovery approached 100% (93 ± 19, n = 5), which is another indication that the sample was not too extensively bleached. The diffusion coefficient was 2.0 ± 1.8 × 10^{-9} cm^2/s. This value is somewhat less than that found in other membranes, but this could be due to the extraordinarily high protein content of mitochondria as compared with other membranes.

DISCUSSION

Mitochondria prepared from the waterbugs showed respiratory control and normal concentrations of the cytochromes. The preparation as isolated contained organelles that were swollen and whose outer membrane was partially damaged: ascorbate oxidation was increased by the addition of cytochrome *c*, which normally does not penetrate intact outer

TABLE III

Recovery and Diffusion Coefficients for Porphyrin Cytochrome c Bound to Mitochondria

Conditions*	n‡	Recovery§	D‖	Mean D¶	ANOV,F Calc.**	ANOV,F Tab.‡‡
		%	$cm^2/s \times 10^{-11}$			
Unwashed	4	93 ± 4	5.8 ± 2.0			
no KCl	8	96 ± 3	5.9 ± 1.3			
	3	75 ± 20	8.3 ± 1.4	6.2 ± 0.4	$F_{0.05(1),5,17}$	
	3	98 ± 6	5.5 ± 2.7			
	2	98 ± 5	5.9 ± 0.5		1.94	2.81
	3	96 ± 2	8.4 ± 1.8			
Washed	6	66 ± 10	19 ± 9			
no KCl	6	57 ± 13	20 ± 5	16 ± 4	$F_{0.05(1),2,13}$	
	4	60 ± 8	8.4 ± 0.6		4.38	3.81
Unwashed	4	85 ± 18	17 ± 14			
25 mM KCl	3	84 ± 8	20 ± 8	16 ± 4	$F_{0.05(1),2,6}$	
	2	99 ± 2	15 ± 4		0.17	5.14
Washed	3	46 ± 6	37 ± 3			
25 mM KCl	3	64 ± 7	28 ± 13	25 ± 7	$F_{0.05(1),3,14}$	
	9	49 ± 7	28 ± 17			
	3	58 ± 12	19 ± 11		0.51	3.34
Unwashed	3	98 ± 2	32 ± 6	33 ± 5	$F_{0.05(1),1,4}$	
50 mM KCl	3§§	96 ± 2	36 ± 12		0.19	7.71
Unwashed	3	98 ± 1	36 ± 17	32 ± 10	$F_{0.05(1),1,4}$	
100 mM KCl	3§§	96 ± 1	30 ± 12		0.22	7.71
Unwashed, uncoupler	3	96 ± 3	17 ± 3	—	—	
Washed, uncoupler	3	69 ± 8	27 ± 10	—	—	

* Mitochondria were incubated with 20 μM porphyrin cytochrome c as described in Materials and Methods except in the case indicated by §§, where the concentration was 40 μM.
‡ Number of measurements on different mitochondria from the same mitochondrial preparation.
§ Mean percent recovery ± SD.
‖ Diffusion coefficient (D) ± SD.
¶ Mean D for all experiments ± SD.
** Analysis of variance (*ANOV*); F is calculated as described (35).
‡‡ F values from tabulated values for $\alpha = 0.05$ (35).

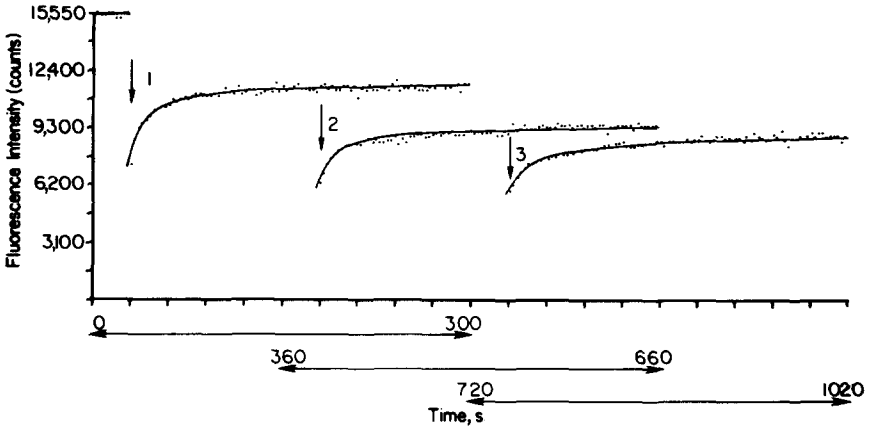

FIGURE 4 FRAP profiles for three consecutive bleaches of porphyrin cytochrome c-labeled mitochondria. Mitochondria (1.5–2.0 mg protein/ml) were incubated with 20 μM porphyrin cytochrome c for 15 min. Unbound porphyrin cytochrome c was removed by centrifugation at 3,600 *g* for 5 min. The pellet was resuspended in 0.5 ml of MSE and FRAP measurements were performed as described in Materials and Methods. The diffusion coefficient, $D = 1.9 \pm 0.5 \times 10^{-10}$ cm²/s and fluorescence recovery: (*a*) 55%; (*b*) 70%; and (*c*) 80%.

membrane. The fragility of the large mitochondria suspended in the hypotonic media did not allow us to use sucrose gradient centrifugation for purification of the mitoplasts. However, we incubated the mitochondria with porphyrin cytochrome *c* for 15 min to allow for its penetration into the intermembrane space through the disrupted outer membrane and its exchange with bound, endogenous cytochrome *c*. Since porphyrin cytochrome *c* is a competitive inhibitor of native cytochrome *c*, we feel that under these conditions at least a part of porphyrin cytochrome *c* was bound at the physiological, high-affinity sites located on the outer surface of the inner mitochondrial membrane.

Under conditions of excess porphyrin cytochrome *c*, our results compare favorably with those obtained by others. Hochman et al. (14) found a diffusion coefficient of 1.6×10^{-10} cm²/s for a 10-fold excess of cytochrome *c* bound to mitochondria obtained from cuprizone-treated mice. Gupte et al. (11) report a value of 6×10^{-11} cm²/s for calcium-fused inner mitochondrial membranes at low ionic strength. These values are not substantially different from the figure of 6.2×10^{-11} cm²/s reported here and obtained under similar experimental conditions. It should be mentioned, that although the specific high-affinity binding sites for cytochrome *c* on the outer surface of the inner mitochondrial membrane should be preferentially filled in our studies, binding to the fragments of the outer membrane can neither be excluded nor reliably quantified. Moreover, in view of the lack of information on the distribution of the marker enzyme for the outer membranes in the preparation of previous investigators (11, 14), it is impossible to evaluate the extent of interference from this source of error in their studies. However, the agreement between our results and those of other authors would suggest that either the nature (i.e., localization) or the properties (i.e., protein, lipid, or both) of the binding sites for fluorescent derivatives of cytochrome *c* were similar in all three studies.

Gupte et al. (11) and we have observed that with increasing ionic strength there is an increase in the apparent diffusion rate for cytochrome *c* derivatives, when they are in excess. The effect of KCl at excess concentrations of porphyrin cytochrome *c* suggests that the apparent diffusion rate is really a reflection of the exchange between bound and free molecules. Further evidence for this suggestion is the finding that at high concentrations of porphyrin cytochrome *c* the apparent diffusion coefficient was independent of porphyrin cytochrome *c* concentration (Table II).

If at high concentrations of porphyrin cytochrome c (>20 μM) the FRAP profile reflects the exchange between free and bound cytochrome, and if the off-rate is solely determining the exchange rate between them, then the recovery profile should fit to a simple exponential (26). We compared curves simulated by Eq. 1 for diffusion and simulated by an exponential for rebinding with representative recovery curves. The shapes of the simulated curves were not sufficiently different to distinguish which better describes the experimental data and so the conclusion that the recovery curves reflect the rebinding of cytochrome *c* cannot be overruled. Therefore, the values for "diffusion" coefficient given in Tables II and III reflect a binding component and are not solely accounted for by diffusion.

Perhaps the most striking observation of this study is that at porphyrin cytochrome *c* concentrations that are about the same as native cytochrome *c* (i.e., 2:1 cytochrome *c* to cytochrome *a*), there is an immobilized fraction. It is reasonable to assume that at these low concentrations the high-affinity sites are predominantly filled. We suggest the following possibilities with respect to the origin of the immobilized fraction:

(*a*) Cytochrome *c* is bound with high affinity to cytochrome *c* oxidase and reductase and these components may be immobilized because they aggregate into large complexes. Consistent with this idea is the finding that only about half of the cytochrome *c* oxidase appears to be rotationally mobile (17, 18). The rotational mobility of zinc cytochrome *c* also showed biphasicity which was interpreted to be due to rotation in a cone, but could also be due to an immobilized fraction (6). Some mobility of the electron-transfer components is, however, suggested by observations indicating the independent diffusion of cytochrome oxidase and cytochrome bc_1 complex (13) and their electrophoretic mobility (25) as well as by electron micrographs of inner mitochondrial membranes labeled with antibodies against oxidase and reductase which show random distribution (12).

(*b*) It is well known that certain plasma membrane proteins diffuse very slowly (or not at all) because of interaction with a supporting system. Interaction of plasma membrane proteins with the cytoskeleton or membrane skeleton in erythrocytes, for example, has been reported (4, 20). In addition, the glycocalyx has been suggested to restrict mobility of plasma proteins (33). In mitochondria, the outer membrane could in effect be an exoskeleton, providing support. Alternatively, inner mitochondrial membrane could also be equipped with some kind of "skeleton." We may point out that nonrandom distribution of the mitochondrial components can be seen in early electron micrographs such as those shown by Fernandez-Moran et al. (9) and Smith (24). It can

be seen (9) that the knobs, associated with the ATPase, are not randomly distributed, but concentrated on the cristae foldings: these observations can be used to argue against free mobility of respiratory chain components.

(*c*) Cytochrome *c* may be mobile within a small domain, but then may hit a barrier. The barrier may arise from several sources. Although we were using swollen mitochondria, we cannot rule out that convolutions of the inner mitochondrial membrane may present a barrier. Other barriers, such as immobilized membrane proteins which would corral cytochrome *c*, are also possible.

The authors thank Dr. David Jameson for setting up the FRAP equipment and Mr. Brian Hill and Mr. Vincent Moy for their help in computer programming. They also thank Dr. David White and Dr. Yale Goldman for assistance in obtaining the waterbugs and for pointing out that these insects contain giant mitochondria.

This work was supported by National Institutes of Health grant HL 18708. A preliminary report of this work was presented to the Biophysical Society, February, 1984 (22).

Received for publication 5 January 1984, and in revised form 9 October 1984.

REFERENCES

1. Axelrod, D., D. E. Koppel, J. Schlessinger, E. Elson, and W. W. Webb. 1976. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* 16:1055–1069.
2. Barisas, B. G., and M. D. Leuther. 1979. Fluorescence photobleaching recovery measurement of protein absolute diffusion constants. *Biophys. Chem.* 10:221–229.
3. Bosshard, H. R., M. Zurrer, H. Schagger, and G. von Jagow. 1979. Binding of cytochrome *c* to the cytochrome bc_1 complex (complex III) and its subunits cytochrome c_1 and *b*. *Biochem. Biophys. Res. Commun.* 89:250–258.
4. Branton, D., C. M. Cohen, and J. Tyler. 1981. Interaction of cytoskeletal proteins on the human erythrocyte membrane. *Cell.* 24:24–32.
5. Dixit, B. P. S. N., and J. M. Vanderkooi. 1984. Probing structure and motion of the mitochondrial cytochromes. *Curr. Top. Bioenerg.* 13:159–202.
6. Dixit, B. P. S. N., A. J. Waring, K. O. Wells III, P. S. Wong, G. V. Woodrow III, and J. M. Vanderkooi. 1982. Rotational motion of cytochrome *c* derivatives bound to membranes measured by fluorescence and phosphorescence anisotropy. *Eur. J. Biochem.* 126:1–9.
7. Erecinska, M., J. S. Davis, and D. F. Wilson. 1980. Interactions of cytochrome *c* with mitochondrial membranes. Binding to succinate-cytochrome *c* reductase. *J. Biol. Chem.* 255:9653–9658.
8. Ferguson-Miller, S., D. F. Brautigan, and E. Margoliash. 1976. Correlation of the kinetics of electron transfer activity of various eukaryotic cytochromes *c* with binding to mitochondrial cytochrome *c* oxidase. *J. Biol. Chem.* 251:1104–1115.
9. Fernandez-Moran, H., T. Oda, P. V. Blair, and D. E. Green. 1964. A macromolecular repeating unit of mitochondrial structure and function. Correlated electron microscopic and biochemical studies of isolated mitochondria and submitochondrial particles of beef heart muscle. *J. Cell Biol.* 22:63–100.
10. Gornall, A. G., C. J. Bardawill, and A. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* 177:751–766.
11. Gupte, S., E.-S. Wu, L. Hochli, M. Hoechli, K. Jacobson, A. E. Sowers, and C. R. Hackenbrock. 1984. Relationship between lateral diffusion, collision frequency, and electron transfer of mitochondrial inner membrane oxidation-reduction components. *Proc. Natl. Acad. Sci. USA.* 81:2606–2610.
12. Hochli, M., and C. R. Hackenbrock. 1979. Lateral translational diffusion of cytochrome *c* oxidase in the mitochondrial energy-transducing membrane. *Proc. Natl. Acad. Sci. USA.* 76:1236–1240.
13. Hochli, M., L. Hochli, and C. R. Hackenbrock. 1981. Independent motional freedom of cytochrome bc_1 and cytochrome oxidase in the inner mitochondrial membrane. *J. Cell Biol.* 91(3, Pt. 2):271*a*. (Abstr.)
14. Hochman, J. H., M. Schindler, J. G. Lee, and S. Ferguson-Miller. 1982. Lateral mobility of cytochrome *c* on intact mitochondrial membranes as determined by fluorescence redistribution after photobleaching. *Proc. Natl. Acad. Sci. USA.* 79:6866–6870.
15. Jacobson, K. 1980. Fluorescence recovery after photobleaching: lateral mobility of lipids and proteins in model membranes and on single cell surfaces. *In* Lasers in Biology and Medicine. F. Hillenkamp, R. Pratesi, and C. A. Sacchi, editors. Plenum Publishing Corp., New York. 271–288.
16. Jacobson, K., Y. Hou, and J. Wojcieszyn. 1978. Evidence for lack of damage during photobleaching measurements of the lateral mobility of cell surface components. *Exp. Cell Res.* 116:179–189.
17. Kawato, S., E. Sigel, E. Carafoli, and R. J. Cherry. 1980. Cytochrome oxidase rotates in the inner membrane of intact mitochondria and submitochondrial particles. *J. Biol. Chem.* 255:5508–5510.
18. Kawato, S., E. Sigel, E. Carafoli, and R. J. Cherry. 1981. Rotation of cytochrome oxidase in phospholipid vesicles. *J. Biol. Chem.* 256:7518–7527.
19. Koppel, D. E. 1979. Fluorescence redistribution after photobleaching. *Biophys. J.* 28:281–291.
20. Koppel, D. E., M. P. Sheetz, and M. Schindler. 1981. Matrix control of protein diffusion in biological membranes. *Proc. Natl. Acad. Sci. USA.* 78:3576–3580.
21. Lehninger, A. L., M. U. Hassan, and H. C. Sudduth. 1954. Phosphorylation coupled to the oxidation of ascorbic acid by isolated mitochondria. *J. Biol. Chem.* 210:911–922.
22. Maniara, G., J. M. Vanderkooi, and M. Erecinska. 1984. Lateral mobility of cytochrome *c* in coupled mitochondria. *Biophys. J.* 45:90*a*. (Abstr.)
23. Schneider, M. B., and W. W. Webb. 1981. Measurement of submicron laser beam radii. *Applied Optics.* 20:1382–1388.
24. Smith, D. S. 1963. The structure of flight muscle sarcosomes in the blowfly *Calliphora erythrocephala* (diptera). *J. Cell Biol.* 19:115–138.
25. Sowers, A. E., and C. R. Hackenbrock. 1981. Rate of lateral diffusion of intramembrane particles: measurement by electrophoretic displacement and rerandomization. *Proc. Natl. Acad. Sci. USA.* 78:6246–6250.
26. Thompson, N. L., T. P. Burghardt, and D. Axelrod. 1981. Measuring surface dynamics of biomolecules by total internal reflection fluorescence with photobleaching recovery or correlation spectroscopy. *Biophys. J.* 33:435–454.
27. Utsumi, K., and L. Packer. 1967. Glutaraldehyde-fixed mitochondria. I. Enzyme activity, ion translocation and conformational changes. *Arch. Biochem. Biophys.* 121:633–640.
28. Vanderkooi, J. M., and M. Erecinska. 1975. Cytochrome *c* interaction with membranes. Absorption and emission spectra and binding characteristics of iron-free cytochrome *c*. Eur. J. Biochem. 60:199–207.
29. Vanderkooi, J., M. Erecinska, and B. Chance. 1973. Cytochrome *c* interaction with membranes. I. Use of a fluorescent chromophore in the study of cytochrome *c* interaction with artificial and mitochondrial membranes. *Arch. Biochem. Biophys.* 154:219–229.
30. Vanderkooi, J. M., P. Glatz, J. Casadei, and G. V. Woodrow III. 1980. Cytochrome *c* interaction with yeast cytochrome b_2. Heme distances determined by energy transfer in fluorescence resonance. *Eur. J. Biochem.* 110:189–196.
31. Vanderkooi, J. M., R. Landesberg, G. W. Hayden, and C. S. Owen. 1977. Metal-free and metal-substituted cytochromes *c*. Use in characterization of the cytochrome *c* binding site. *Eur. J. Biochem.* 81:339–347.
32. Wojtczak, L., and G. L. Sottocasa. 1972. On the impermeability of the outer mitochondrial membrane to cytochrome *c*. *J. Membr. Biol.* 7:313–324.
33. Wolf, D. E., P. Henkar, and W. W. Webb. 1980. Diffusion, patching and capping of stearoylated dextrans on 3T3 cell plasma membranes. *Biochemistry.* 19:3893–3903.
34. Yguerabide, J., J. A. Schmidt, and E. E. Yguerabide. 1982. Lateral mobility in membranes as detected by fluorescence recovery after photobleaching. *Biophys. J.* 39:69–75.
35. Zar, J. H. 1974. Biostatistical Analysis. Prentice Hall, Englewood Cliffs, NJ. 134 and 415–449.